

RAPID COMMUNICATION

Adenoviral E1A Protein Nuclear Import Is Preferentially Mediated by Importin $\alpha 3$ *in Vitro*

M. Köhler,* D. Görlich,† E. Hartmann,‡ and J. Franke*

*HELIOS Clinic/Franz Volhard Clinic/Max Delbrueck Center, Humboldt University Berlin, 13122 Berlin; †Center for Molecular Biology Heidelberg, University of Heidelberg, Heidelberg; and ‡Institute of Biology, University of Luebeck, Luebeck, Germany

Received June 21, 2001; returned to author for revision July 30, 2001; accepted August 17, 2001

Nuclear import of some viral proteins depends on importin α proteins. However, no preferences of distinct α -importins for any viral protein import have been demonstrated. We used *in vitro* import assays and observed that all ubiquitously expressed human importin α isoforms mediate nuclear translocation of adenoviral E1A. Competition with nucleoplasmin suggests that importin $\alpha 3$ is the most efficient import mediator of E1A. © 2001 Academic Press

Introduction. Nuclear translocation of viral proteins is crucial for their specific functions. Like for other nuclear proteins, their transport across the nuclear pore complexes is generally active if they are larger than 20–30 kDa and requires both specific signals within these molecules and distinct soluble transport factors (for review see (5)). Several pathways mediate the import of macromolecules into the nucleus. Members of the importin β -like receptors are usually involved. The common feature of these receptors is the presence of a RanGTP-binding domain. During import, the receptor binds its cargo in the cytosol, an area of low RanGTP, and carries it through the nuclear pore. In the RanGTP-rich nucleoplasm, the import receptor binds to RanGTP, thereby triggering the release of its cargo. In some import pathways, importin β or another family member alone is sufficient to promote import. Other pathways rely on the presence of two different importin β -like molecules. Still others need an adaptor protein that links the cargo with the receptor. Most cellular proteins use the “classical” nuclear protein import pathway that is dependent on distinct nuclear localization signal sequences (NLSs) (for review see (6)). Here, the proteins bind to their receptor importin β not directly, but via importin α s (also termed karyopherin α or PTAC 58). Humans have one importin β but six importin α isoforms (8, 9, 12, 19). The various importin α isoforms are differentially expressed in various tissues and cells. However, whereas importins $\alpha 1$ /Rch1, $\alpha 3$ /Qip1, $\alpha 4$ /hSRP1 γ , $\alpha 5$ /hSRP1, and $\alpha 7$ are ubiquitously expressed in various cells and tissues, importin $\alpha 6$ has been detected at the mRNA level only in the testis (7–9, 12, 16). The α importins appear to differ in their substrate-specific import efficiencies, which may provide an additional step for the control of the intracellular localization of proteins (9, 11, 12, 14, 20). Many viral

proteins translocate into the nucleus via importin β without importin α or via unknown pathways. Other viral proteins use the classical importin α/β -dependent pathway (for review see (21)). However, to our knowledge, no import preferences of distinct importin α isoforms for any viral proteins have been demonstrated so far. We addressed the question of whether nuclear import of viral proteins can be mediated preferentially by a distinct importin α protein, as has been observed for mammalian proteins using adenoviral E1A as a model. Although the soluble factors mediating nuclear import of E1A have not been elucidated, a NLS that is both necessary and sufficient for E1A nuclear protein import located at the carboxy terminus is known (10). A second E1A-NLS that has been found to function in *Xenopus* oocytes is silent in somatic cells (15). Here we demonstrate that all ubiquitously expressed importin α proteins can mediate nuclear translocation of an E1A-NLS fusion protein *in vitro*. However, competition experiments with nucleoplasmin suggest that importin $\alpha 3$ is the most potent import mediator of E1A.

Results and Discussion. To investigate E1A nuclear import *in vitro* we used a fluorescein-labeled fusion of the carboxy terminal NLS to BSA that has been characterized by Cordes *et al.* (1). We performed *in vitro* nuclear import assays analyzing all ubiquitously expressed importin α isoforms, namely importins $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 7$, as described previously (9). All importin α proteins tested mediated nuclear translocation of E1A-NLS-BSA without major differences in the presence of importin β (Fig. 1A). When no importin α was added to the import reaction, no labeled E1A-NLS-BSA was detected in the nuclei of permeabilized cells. When nucleoplasmin was added as a second substrate to the import reaction, a

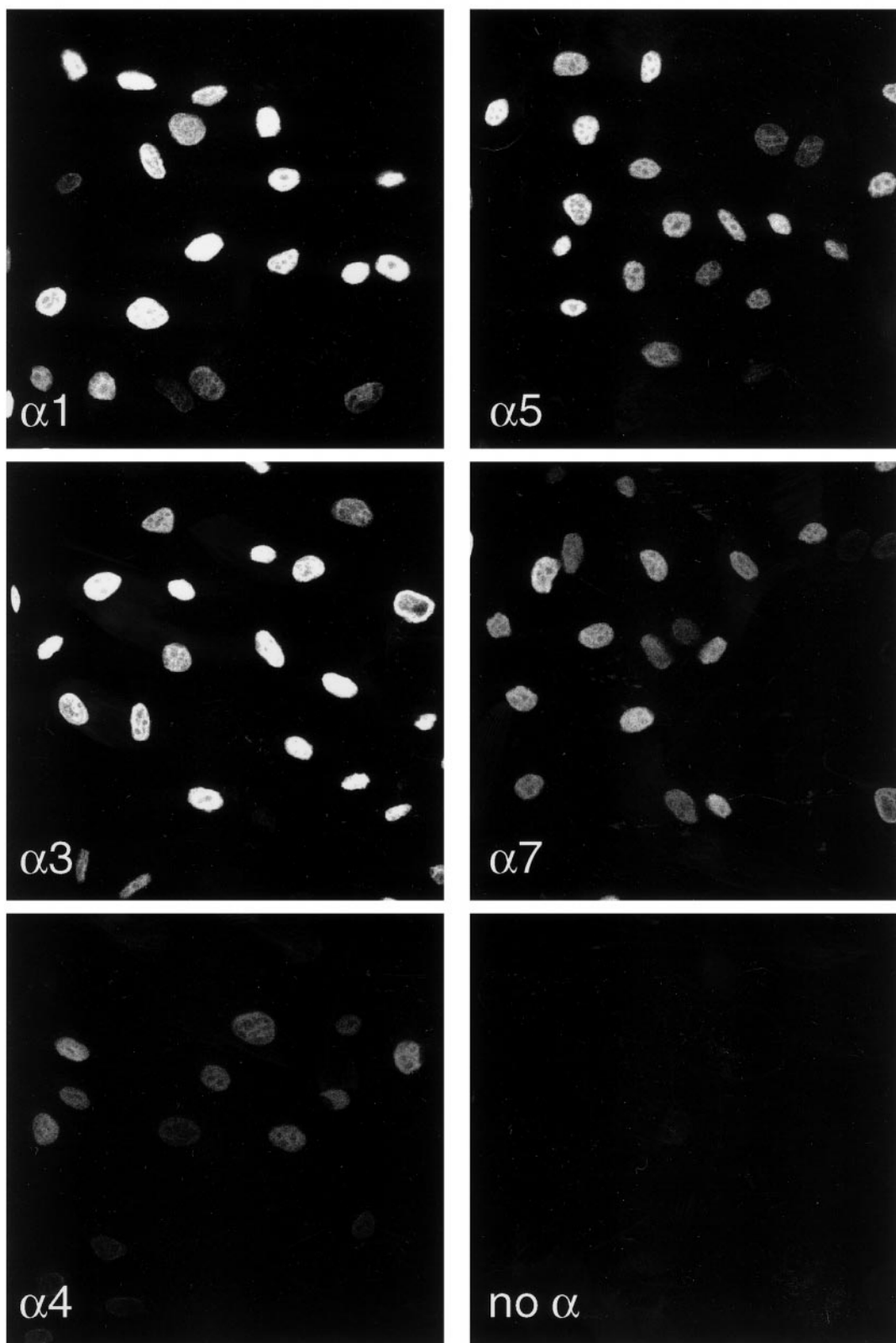
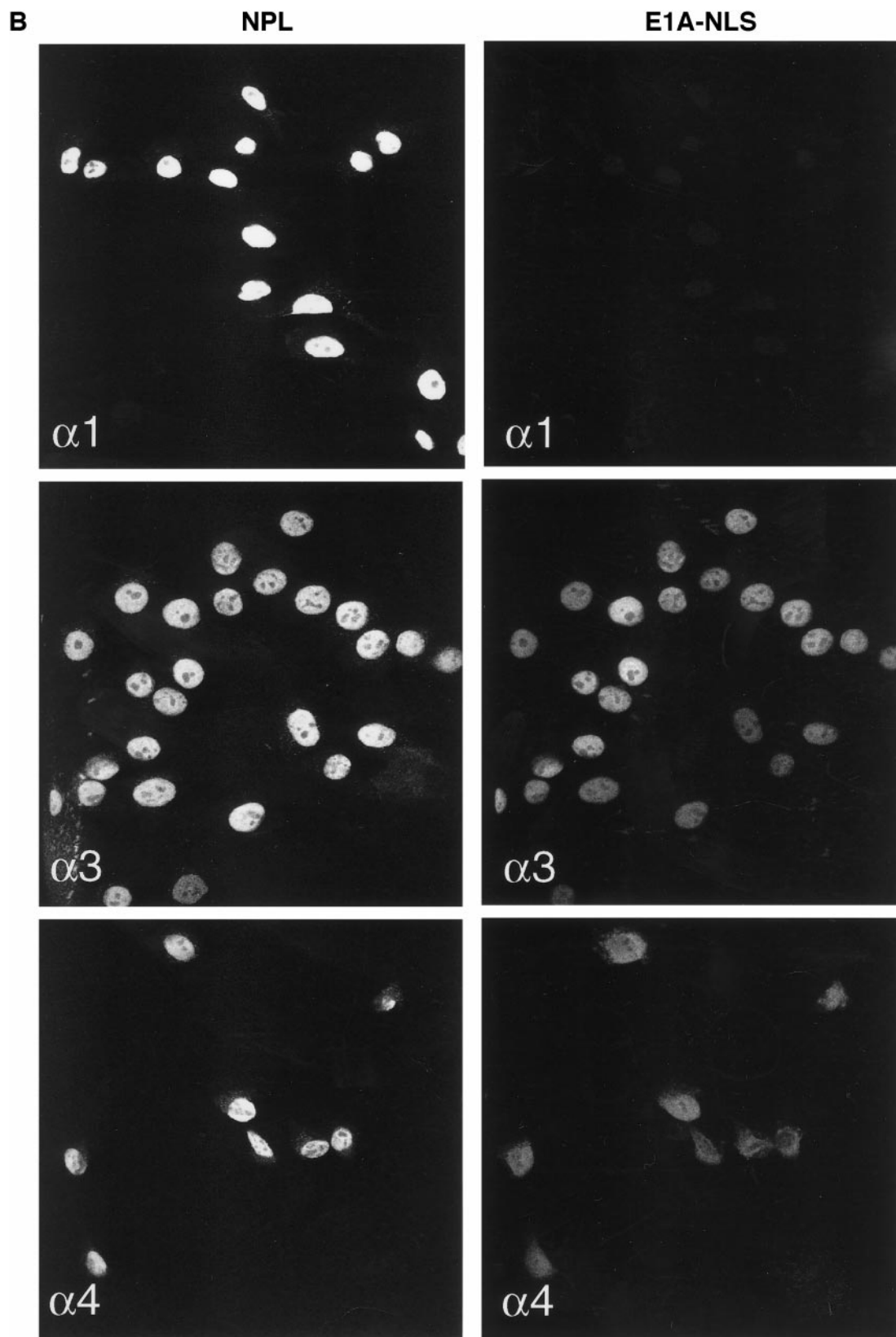
A**E1A-NLS**

FIG. 1. Importin α -dependent nuclear import of adenoviral E1A-NLS-BSA protein in permeabilized HeLa cells. HeLa cells were grown on slides, permeabilized, and incubated with the import mixture. Reactions were stopped by fixation with 4% paraformaldehyde and coverslips were analyzed by confocal microscopy. The import reactions consisted of an energy-regenerating system, nucleoplasmin core buffer, reticulocyte lysate, and purified transport factors (RanGDP, Rna1p, RanBP1, NTF2, importin β , and the indicated importin α protein). Identical exposure times for each α -importin and negative control were used. (A) FITC-labeled E1A-NLS-BSA was added as the only substrate to the import reaction. (B) Texas Red-labeled nucleoplasmin (left) and FITC-labeled E1A-NLS-BSA (right) were both added for competition to the import reaction. $\alpha 1$, importin $\alpha 1$ /Rch1; $\alpha 3$, importin

FIG. 1—*Continued*

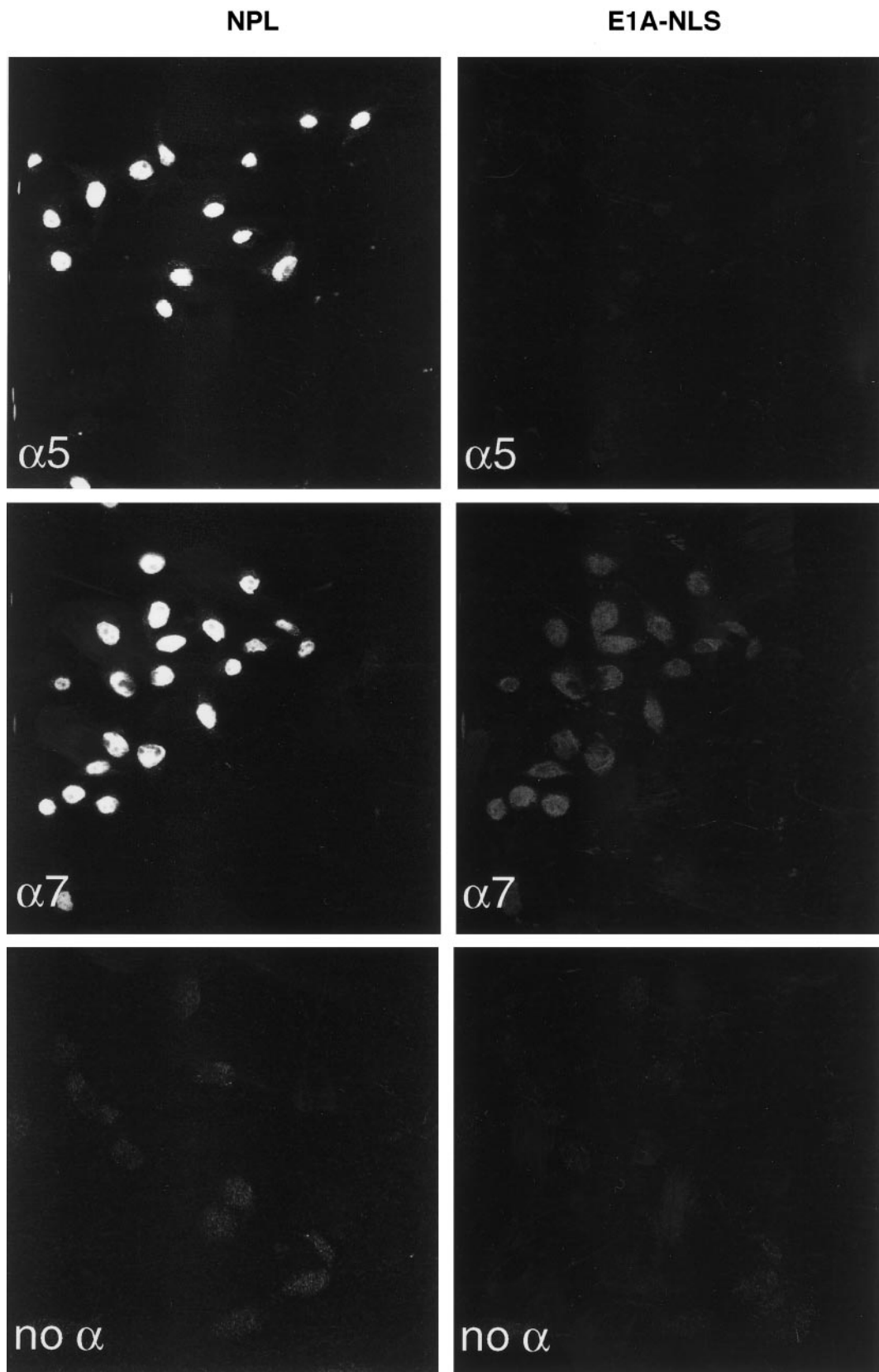


FIG. 1—Continued

strong inhibition of E1A nuclear accumulation for almost all importin α isoforms tested was observed (Fig. 1B). There were no differences between importins $\alpha 1$ and $\alpha 5$ and the negative control without any importin α added. Weak nuclear import of E1A in the presence of nucleoplasmin was detected for importins $\alpha 4$ and $\alpha 7$. Only importin $\alpha 3$ continued to mediate strong nuclear translocation of E1A. One could argue that the observed differences in E1A import efficiencies of the distinct α importins might be entirely due to varying relative affinities of nucleoplasmin for the α importins. However, we have shown previously that importin $\alpha 3$ is more efficient in nuclear transport of nucleoplasmin than importins $\alpha 1$ and $\alpha 7$ *in vitro* (4, 9). Furthermore, we also reported that nucleoplasmin shows no significant binding differences to the various importin α forms (9). Therefore, our data indicate that nuclear import of adenoviral E1A protein can be mediated by the classical importin α/β -dependent import pathway and that importin $\alpha 3$ is likely to be the most efficient import mediator for this substrate. Previously, binding studies analyzing only two of the different importin α isoforms suggested different efficiencies regarding nuclear translocation of the hepatitis δ antigen (2). However, to our knowledge, no substrate-specific differences of the transport efficiencies of the importin α proteins have been reported for other viral proteins. Interestingly, one of the nuclear E1A interacting proteins, namely P/CAF, is also translocated into the nucleus preferentially by importin $\alpha 3$ (9). Not all viral proteins that are imported via importin α/β necessarily display strong preferences toward a particular importin α isoform. Nuclear import of influenza viral (NP) protein has been shown to be mediated by importins $\alpha 1$ and $\alpha 5$ (13). Using a maltose-binding NP fusion protein described by Elton *et al.* we found that all importin α s can mediate nuclear import of NP *in vitro* (3). However, we could not detect a strong preference of a distinct importin α protein for NP (data not shown). Whether this result is due to the reported presence of several NLSs in the NP protein remains unresolved (17, 18). The specificity of E1A import suggests a special need for regulating its nuclear import.

Materials and Methods. *In vitro* nuclear import assays were performed as described previously (9). Briefly, HeLa cells were grown on microscopy slides (Roth) to 40–80% confluence, washed once in ice-cold phosphate-buffered saline (PBS), and permeabilized for 8 min with 30 μ g/ml digitonin (Sigma). Permeabilized cells were incubated with 20 μ l of import mixture for 8 min at room temperature. The import reactions were stopped by fixation with 4% paraformaldehyde in PBS and the slides were analyzed by confocal microscopy (MRC 1024, Bio-Rad). The import reactions consisted of an energy-regenerating system, core buffer, EGTA, 10% reticulocyte lysate, and the import factors RanGDP, Rna1p, RanBP1, NTF2, importin β , and one of the importin α proteins. Preparation and

fluorescence labeling of recombinant proteins has been described (9).

ACKNOWLEDGMENTS

We thank B. Nentwig for excellent technical help. We are grateful to T. Sommer, C. Quensel, K. Stade, and J. Mymryk for fruitful discussions and helpful comments. The E1A-NLS-BSA and maltose-binding NP fusion proteins were kind gifts of V. Cordes and D. Elton. Finally, we are grateful to F. C. Luft for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (DFG KO 1950/1-3) to M.K. and the Fond der Chemischen Industrie to E.H.

REFERENCES

1. Cordes, V. C., Rackwitz, H. R., and Reidenbach, S. (1997). Mediators of nuclear protein import target karyophilic proteins to pore complexes of cytoplasmic annulate lamellae. *Exp. Cell Res.* **237**, 419–433.
2. Chou, H.-C., Hsieh, T.-Y., Sheu, G.-T., and Lai, M. M. (1998). Hepatitis delta antigen mediates the nuclear import of hepatitis delta virus RNA. *J. Virol.* **72**, 3584–3690.
3. Elton, D., Simpson-Holley, M., Archer, K., Medcalf, L., Hallam, R., McCauley, J., and Digard, P. (2001). Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway. *J. Virol.* **75**, 408–419.
4. Franke, J., Reimann, B., Hartmann, E., Kohler, M., and Wiedmann, B. (2001). Evidence for a nuclear passage of nascent polypeptide-associated complex subunits in yeast. *J. Cell Sci.* **114**, 2641–2648.
5. Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **15**, 607–660.
6. Jans, D. A., Xiao, C. Y., and Lam, M. H. (2000). Nuclear targeting signal recognition: A key control point in nuclear transport? *Bioessays* **22**, 532–544.
7. Kamei, Y., Yuba, S., Nakayama, T., and Yoneda, Y. (1999). Three distinct classes of the alpha-subunit of the nuclear pore-targeting complex (importin-alpha) are differentially expressed in adult mouse tissues. *J. Histochem. Cytochem.* **47**, 363–372.
8. Kohler, M., Ansieau, S., Prehn, S., Leutz, A., Haller, H., and Hartmann, E. (1997). Cloning of two novel human importin-alpha subunits and analysis of the expression pattern of the importin-alpha protein family. *FEBS Lett.* **417**, 104–108.
9. Kohler, M., Speck, C., Christiansen, M., Bischoff, F. R., Prehn, S., Haller, H., Gorlich, D., and Hartmann, E. (1999). Evidence for distinct substrate specificities of importin alpha family members in nuclear protein import. *Mol. Cell. Biol.* **19**, 7782–7791.
10. Lyons, R. H., Ferguson, B. Q., and Rosenberg, M. (1987). Pentapeptide nuclear localization signal in adenovirus E1a. *Mol. Cell. Biol.* **7**, 2451–2456.
11. Miyamoto, Y., Imamoto, N., Sekimoto, T., Tachibana, T., Seki, T., Tada, S., Enomoto, T., and Yoneda, Y. (1997). Differential modes of nuclear localization signal (NLS) recognition by three distinct classes of NLS receptors. *J. Biol. Chem.* **272**, 26375–26381.
12. Nachury, M. V., Ryder, U. W., Lamond, A. I., and Weis, K. (1998). Cloning and characterization of hSRP1 gamma, a tissue-specific nuclear transport factor. *Proc. Natl. Acad. Sci. USA* **95**, 582–587.
13. O'Neill, R. E., Jaskunas, R., Blobel, G., Palese, P., and Moroianu, J. (1995). Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. *J. Biol. Chem.* **270**, 22701–22704.
14. Sekimoto, T., Imamoto, N., Nakajima, K., Hirano, T., and Yoneda, Y. (1997). Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. *EMBO J.* **16**, 7067–7077.
15. Slavicek, J. M., Jones, N. C., and Richter, J. D. (1989). A karyophilic

- signal sequence in adenovirus type 5 E1A is functional in *Xenopus* oocytes but not in somatic cells. *J. Virol.* **63**, 4047–4050.
16. Tsuji, L., Takumi, T., Imamoto, N., and Yoneda, Y. (1997). Identification of novel homologues of mouse importin alpha, the alpha subunit of the nuclear pore-targeting complex, and their tissue-specific expression. *FEBS Lett.* **416**, 30–34.
17. Wang, P., Palese, P., and O'Neill, R. E. (1997). The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza A virus nucleoprotein NP is a nonconventional nuclear localization signal. *J. Virol.* **71**, 1850–1856.
18. Weber, F., Kochs, G., Gruber, S., and Haller, O. (1998). A classical bipartite nuclear localization signal on Thogoto and influenza A virus nucleoproteins. *Virology* **250**, 9–18.
19. Weis, K., Mattaj, J. W., and Lamond, A. I. (1995). Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. *Science* **268**, 1049–1053.
20. Welch, K., Franke, J., Kohler, M., and Macara, I. G. (1999). RanBP3 contains an unusual nuclear localization signal that is imported preferentially by importin-alpha3. *Mol. Cell. Biol.* **19**, 8400–8411.
21. Whittaker, G. R., Kann, M., and Helenius, A. (2000). Viral entry into the nucleus. *Annu. Rev. Cell Dev. Biol.* **16**, 627–651.